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Abbreviate title: Sex dependent C fibre ADS in inflammatory pain

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Abstract

C fibres display activity-dependent slowing (ADS), whereby repetitive stimulation (≥1Hz) results in a progressive slowing of action potential conduction velocity, which manifests as a progressive increase in response latency. However, the impact of ADS upon spinal pain processing has not been explored nor whether ADS is altered in inflammatory pain conditions. To investigate, compound action potentials were made, from dorsal roots isolated from rats with or without complete Freund's adjuvant (CFA) hindpaw inflammation, in response to electrical stimulus trains. CFA inflammation significantly reduced C fibre ADS at 1 and 2Hz stimulation rates. Whole-cell patch-clamp recordings in the spinal cord slice preparation with attached dorsal roots, also demonstrated that CFA inflammation reduced ADS in the monosynaptic C fibre input to lamina I neurokinin 1 receptor expressing neurons (1-10Hz stimulus trains) without altering the incidence of synaptic response failures. When analysed by sex it was revealed that females display a more pronounced ADS that is reduced by CFA inflammation to a level comparable with males. Cumulative ventral root potentials evoked by long and short dorsal root stimulation lengths, to maximise and minimise the impact of ADS, respectively, demonstrated that reducing ADS facilitates spinal summation and this was also sex-dependent. This finding correlated with the behavioural observation of increased noxious thermal thresholds and enhanced inflammatory thermal hypersensitivity in females. We propose that sex/inflammation dependent regulation of C fibre ADS can, by controlling the temporal relay of nociceptive inputs influence the spinal summation of nociceptive signals contributing to sex/inflammation dependent differences in pain sensitivity.

Significance Statement

The intensity of a noxious stimulus is encoded by the frequency of action potentials relayed by nociceptive C fibres to the spinal cord. C fibres conduct successive action potentials at progressively slower speeds but the impact of this activity-dependent slowing (ADS) is unknown. Here we demonstrate that ADS is more prevalent in females than males and is reduced in an inflammatory pain model in females only. We also demonstrate a progressive delay of C fibre monosynaptic transmission to the spinal cord that is similarly sex and inflammation dependent. Experimentally manipulating ADS strongly influences spinal summation consistent with sex differences in behavioural pain thresholds. This suggests that ADS provides a peripheral mechanism that can regulate spinal nociceptive processing and pain sensation.

Introduction

The basic currency of communication in the nervous system is the action potential. As the action potential is an all-or-none event, information is coded by the number of action potentials and the time intervals between them. In the 1920's Edgar Adrian demonstrated that the intensity of a sensation is coded by the firing frequency in afferent nerve fibres in the somatosensory system (Adrian, 1926; Adrian and Zotterman, 1926a, b). Subsequently, microneurography studies in humans have directly demonstrated that the firing frequency in C fibre nociceptors encodes pain intensity (Torebjork et al., 1984; Yarnitsky and Ochoa, 1990). The more noxious the stimulus the shorter the intervals between successive action potentials in nociceptive C fibres and the more intense the pain experienced.

Nociceptive C fibres display *activity-dependent slowing* (ADS), whereby repetitive stimulation results in a progressive slowing of action potential conduction velocity, which manifests as a progressive increase in response latency in both human (Serra et al., 1999; Weidner et al., 1999) and animal studies (Thalhammer et al., 1994; Gee et al., 1996). Subtypes of C fibres display this phenomenon to differing degrees such that ADS can be used to functionally classify C fibre subtypes (Thalhammer et al., 1994; Serra et al., 1999; Weidner et al., 1999). ADS occurs in a frequency and length-dependent manner, with greater ADS observed at higher frequencies (Thalhammer et al., 1994; Gee et al., 1996; Serra et al., 1999; Weidner et al., 1999) and over longer lengths (Schmelz et al., 1995; Zhu et al., 2009). This progressive slowing of conduction velocity presumably regulates the intervals between successive action potentials reaching the spinal cord which could influence central pain processing and pain sensation, however, this has not been investigated.

ADS involves voltage-gated sodium (Nav) channels (De Col et al., 2008; Obreja et al., 2012) likely Nav1.7 and Nav1.8 (Baker and Waxman, 2012; Petersson et al., 2014; Tigerholm et al., 2014; Hoffmann et al., 2016) and is constrained by hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels (Takigawa et al., 1998; Zhu et al., 2009; Mazo et al., 2013). These channels are known to be regulated in inflammatory pain (Emery et al., 2012; Weng et al., 2012; Rahman and Dickenson, 2013; Waxman and Zamponi, 2014) suggesting that C fibre ADS may be altered in inflammatory pain. Specifically, CFA inflammation increases both hyperpolarisation-activated current (I_h) and HCN2 expression levels in C fibre nociceptors (Papp et al., 2010; Acosta et al., 2012; Weng et al., 2012) and alters I_h activation properties (Djouhri et al., 2015). Furthermore, deletion of HCN2 in Nav1.8 expressing neurons, which are mainly C fibre nociceptors limits inflammatory thermal

hyperalgesia (Emery et al., 2011) and peripheral block of HCN channels attenuates inflammatory pain (Young et al., 2014). There is also a loss of inflammatory pain phenotype in Nav1.7 and Nav1.8 knockout mice and in mice in which Nav1.7 was selectively deleted in Nav1.8 expressing neurons (Akopian et al., 1999; Nassar et al., 2004; Nassar et al., 2005). Furthermore, Nav1.7 and Nav1.8 channel expression is increased in CFA inflammation (Coggeshall et al., 2004; Gould et al., 2004; Liang et al., 2013) and selective blockers of either channel reduce inflammatory pain (McGowan et al., 2009; Zhang et al., 2010; Bregman et al., 2011; Yang et al., 2013; Lee et al., 2014; Payne et al., 2015).

The aim of this study was therefore to determine whether C fibre ADS is altered in the CFA inflammation model and the impact upon temporal relay of nociceptive input to the spinal cord. Given increasing awareness of sex differences in pain sensitivity and injury-induced hypersensitivity (Mogil and Bailey, 2010; Mogil, 2012; Bartley and Fillingim, 2013) this was also investigated in both sexes. Furthermore, the impact of ADS upon spinal summation and output was explored using electrophysiological and behavioural analysis of the nociceptive flexion withdrawal reflex.

Materials and Methods

Animals

All experiments were approved by the University of Edinburgh Ethical Review Committee and were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. Sprague Dawley rats of both sexes (the University of Edinburgh Biological Research Resources, Edinburgh UK), were used in all

experiments. Animals were housed in cages at 21°C and 55% relative humidity, with a 12 h light-dark cycle, food and water were provided *ad libitum*.

Inflammatory pain model

To induce peripheral inflammation, juvenile rats received an intraplantar injection of complete Freund's adjuvant (CFA, 0.5 mg/ml saline) into the left hindpaw (1µl/g body weight) under isoflurane anaesthesia, at ~postnatal day (P)18, 2-5 days prior to patch-clamp or compound action potential electrophysiological recording at ~P21. This procedure results in persistent peripheral hindpaw inflammation and behavioural hypersensitivity in rats of this age (Torsney, 2011). Control rats were untreated.

Isolated dorsal root preparation

Isolated dorsal roots were prepared as described previously (Torsney, 2011; Dickie and Torsney, 2014). Briefly, naïve untreated (control) or CFA treated rats (~P21) were decapitated under isoflurane anaesthesia and spinal cords, with attached dorsal roots, were removed in ice-cold dissection solution. Lumbar (L4/L5) dorsal roots (left side only, CFA treated) were cut near the dorsal root entry zone and their dorsal root ganglia were removed, before being placed in 36–37°C oxygenated recovery solution for 1 hour. Roots were transferred to the recording chamber of an upright microscope (Ziess) and were perfused with oxygenated Krebs' solution (1-2ml/min) at room temperature. The 95% O_2/5% CO_2 saturated Krebs' solution contained (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose, 1 $MgCl_2$, and 2 $CaCl_2$, pH 7.4. Recovery solution was identical to Krebs' apart from 1.5mM $CaCl_2$ plus 6mM $MgCl_2$. Dissection solution was the same as recovery, but with 1mM kynurenic acid.

Compound action potential (CAP) recording

Two glass suction electrodes were used, one for electrical orthodromic stimulation and the second for recording compound action potentials. Dorsal roots were stimulated 10 times at 0.2Hz (0.1ms duration), with an ISO-flex stimulus isolator (A.M.P.I. Intracel) at (in μA): 1, 2, 3, 4, 5, 7.5, 10, 15, 20, 25, then in 10μA steps between 30 and 100μA, then in 50μA steps between 150 and 500μA (Torsney, 2011; Dickie and Torsney, 2014). An 0.1ms pulse width was chosen to replicate the electrical stimuli previously established to activate the different afferent fibre types in this age of rat (Nakatsuka et al., 2000). However, the possibility of an underestimation of the C fibre contribution cannot be excluded given that longer pulse widths have also been employed to stimulate C fibre inputs (Baba et al., 1999). The main components of the compound action potentials were differentiated as Aβ, Aδ and C fibre, on the basis of activation threshold and conduction velocity, each displaying a characteristic triphasic (positive-negative-positive) response. Data were acquired and recorded using a Cygnus ER-1 differential amplifier (Cygnus Technologies Inc.) and pClamp 10 software (Molecular Devices). Data were filtered at 10kHz and sampled at 50kHz.

Activation threshold was defined as the lowest stimulation intensity at which the negative component of the triphasic response was clearly identifiable. The amplitude of each component was calculated by measuring the distance between the negative and second positive peak. The conduction velocity was calculated based on the latency to the negative peak at 20, 100 and 500μA for Aβ, Aδ and C components, respectively.

To assess ADS, dorsal roots were stimulated 16 or 40 times (500μA intensity, 0.1ms duration) at frequencies of 1 or 2Hz. For each stimulus, the latency between the

stimulus artefact and the negative peak of the triphasic response was measured and the change in latency from stimulus 1 calculated. In some cases the width of the C fibre component (positive peak to positive peak) was additionally measured and the change in width from stimulus 1 calculated. To negate any influence of varying dorsal root length, the latency/width change was normalised to the length of root stimulated, measured as the distance between the stimulating and recording electrodes. In a subset of recordings the stimulating electrode was first placed close to the distal end of the dorsal root (long stimulation length) and then placed closer to the recording electrode (short stimulation length). By subtracting the latency change values for 'short stimulation length' from 'long stimulation length' the latency change solely attributable to conduction velocity slowing, independent of action potential initiation was calculated.

Spinal cord slice preparation

Spinal cords with attached dorsal roots, from which dorsal root ganglia were removed, were obtained from control or CFA treated (~P21) rats, as described above. The lumbar (L4/L5) segment was embedded in an agarose block and 350μm slices, with attached dorsal roots (left side only, CFA treated), were cut. Slices were placed in oxygenated recovery solution at 36–37°C for 1 hour and were then incubated at room temperature for 30 mins with 35nM tetramethylrhodamine conjugated substance P (TMR-SP), as described previously (Labrakakis and MacDermott, 2003; Torsney, 2011; Dickie and Torsney, 2014). Slices were allowed to recover for a further 1 hour at room temperature before being transferred to the recording chamber of an upright microscope (Ziess), equipped with fluorescence for the identification of TMR-SP labelled (TMR-SP+) neurons and infrared-differential

196 interference contrast (IR-DIC) for electrophysiological recordings, and were
197 continually perfused with oxygenated Krebs (1-2ml/min) at room temperature.

198 *Patch-clamp recording*

199 Whole-cell patch-clamp recordings (holding potential -70mV) were made from TMR-
200 SP+ neurons in the lamina I region of the dorsal horn. The intracellular solution used
201 was composed of (in mM); 120 Cs-methylsulfonate, 10 Na-methylsulfonate, 10
202 EGTA, 1 $CaCl_2$, 10 HEPES, 5 QX-314-Cl [N-(2,6-
203 Dimethylphenylcarbamoylmethyl)triethylammonium chloride] and 2 Mg^{2+}-ATP, pH
204 adjusted to 7.2 with CsOH, osmolarity 290mOsm and junction potential was
205 corrected prior to recording. Additionally, 1μM Alexa Fluor 488 hydrazide was
206 included in the recording pipette. Data were recorded and acquired with an Axopatch
207 200B amplifier and pClamp 10 software (Molecular Devices). Data were filtered at
208 5kHz and digitised at 10kHz.

209

210 Monosynaptic primary afferent input to lamina I NK1R+ neurons was identified as
211 described previously (Torsney and MacDermott, 2006; Torsney, 2011; Dickie and
212 Torsney, 2014). eEPSCs were recorded in response to low frequency (0.05Hz)
213 dorsal root stimulation (3 times) at intensities of 20, 100 and 500μA (0.1ms stimulus
214 duration) to activate Aβ, Aδ and C fibre inputs, respectively, using an ISO-Flex
215 stimulus isolator. To characterise an input as monosynaptic or polysynaptic, dorsal
216 roots were stimulated (20 times) at the following intensities and frequencies; Aβ
217 20μA / 20Hz; Aδ 100μA / 2Hz; C 500μA / 1Hz. A-fibre responses were considered
218 monosynaptic if they displayed no synaptic failures and a stable latency (≤2ms),

while C fibre inputs were considered monosynaptic if they displayed no synaptic failures, regardless of whether there was latency variability (Nakatsuka et al., 2000).

To assess whether monosynaptic Aδ or monosynaptic C fibre input to lamina I NK1R+ neurons displayed ADS in response to repetitive stimulation, eEPSCs were recorded in response to trains of 16 stimuli delivered at 1 or 2Hz (Aδ and C) or trains of 40 stimuli at 2, 5 or 10Hz (C only), at intensities of 100 (Aδ) or 500μA (C). The latency of each eEPSC was measured as the time between the stimulus artefact and the onset of the monosynaptic response and the change in latency from stimulus 1 calculated. These latency change data were also normalised to dorsal root length, measured as the distance between the stimulating electrode and the dorsal root entry zone, to account for variations in the length of dorsal root stimulated.

Dorsal root – ventral root potential (DR-VRP) recording

Control rats (~P10) were decapitated under isoflurane anaesthesia and spinal cords, with attached dorsal and ventral roots, were removed in ice-cold dissection solution. Hemisected lumbar spinal cord with only L4/L5 dorsal and ventral roots left attached were prepared (Otsuka and Konishi, 1974) and transferred to a recording chamber perfused with oxygenated Krebs' solution (>2ml/min) at room temperature. Two glass suction electrodes were used, one for electrical stimulation of the dorsal root and the second for recording ventral root potentials. The stimulating electrode was first placed close to the distal end of the dorsal root (long stimulation length/more ADS) and then placed closer to the spinal cord (short stimulation length/less ADS) to assess the impact of length-dependent ADS upon spinal summation. The dorsal root was stimulated 40 times at 2, 5 and 10Hz (500μA intensity, 0.1ms duration) at both

sites and the cumulative ventral root potential recorded using a close fitting glass electrode placed on the ventral root close to the ventral horn (Thompson et al., 1994). Data were acquired and recorded using a Cygnus ER-1 differential amplifier (Cygnus Technologies Inc.) and pClamp 10 software (Molecular Devices). Data were filtered at 10kHz and sampled at 50kHz.

Sensory testing

Prior to and 2-5 days following intraplantar CFA injection (as detailed above) hindpaw swelling, mechanical and thermal sensitivity were measured. Hindpaw swelling was assessed by measuring the thickness of the dorsal-ventral paw using Vernier calipers. Following habituation on an elevated mesh platform the mechanical threshold of the nociceptive flexion withdrawal reflex was determined with von Frey filaments (Stoelting) applied to the mid-plantar surface of the hindpaw using the up-down method (Chaplan et al., 1994). After habituation to the Hargreaves apparatus, radiant heat was applied to the mid-plantar surface of the hindpaw (x3 stimuli per hindpaw to calculate average) to determine the noxious thermal withdrawal latency.

Statistical analysis

Area under the curve (AUC) analysis was used to compare ADS in both CAP and patch-clamp recordings. Group comparisons, for both electrophysiology and behavioural data, were carried out using 2-way ANOVA with or without repeated measures analysis as appropriate and was followed by Sidak's multiple comparisons test or Tukey's multiple comparisons test, respectively, if an interaction between factors was observed. Averaged data are represented as mean $\pm$ SE.

Materials

All chemicals were obtained from Sigma, except; TMR-SP (Enzo Life Sciences), Alexa Fluor 488 hydrazide (Molecular Probes), QX-314-Cl (Alomone Labs) and NBQX (Tocris Bioscience).

Results

CFA inflammation reduces C fibre ADS in isolated dorsal roots

To assess the impact of CFA inflammation on C fibre ADS, dorsal roots isolated from control or CFA treated rats were repetitively stimulated and the response latencies of Aβ, Aδ and C fibre compound action potentials were measured. The Aβ, Aδ and C fibre afferent components were identified on the basis of conduction velocity and activation threshold (Fig. 1A). CFA inflammation did not alter threshold stimulus intensity (P=0.394: Aβ control (n=10), 7.6±0.80 μA; Aβ CFA (n=12), 5.9±0.60 μA; Aδ control (n=11), 38.6±3.0 μA; Aδ CFA (n=13), 34.6±2.8 μA; C control (n=11), 240.9±14.8 μA; C CFA (n=13) 226.9±15.6 μA) conduction velocity (P=0.418: Aβ control, 4.6±0.50 m/s; Aβ CFA, 5.0±0.30 m/s; Aδ control, 0.9±0.1 m/s; Aδ CFA, 0.7±0.1 m/s; C control, 0.2±0.01 m/s; C CFA 0.2±0.01 m/s) or amplitude (P=0.091: Aβ control, 1.7±0.2 mV; Aβ CFA, 2.4±0.40 mV; Aδ control, 0.1±0.02 mV; Aδ CFA, 0.1±0.02 mV; C control, 0.1±0.02 mV; C CFA 0.1±0.03 mV), as previous studies have demonstrated in both adult (Baba et al., 1999; Nakatsuka et al., 2000) and similarly aged juvenile rats (Torsney, 2011).

Repetitive stimulation of isolated dorsal roots produced a negligible reduction (speeding) in the latency of the Aβ fibre response (Fig. 1Bi,Ci) and a marginal

289 increase (slowing) in the Aδ fibre response latency (Fig. 1Bii,Cii). CFA inflammation
290 did not alter these observations in A fibres (Aβ: P=0.693; Aδ: P=0.451). In contrast,
291 repetitive stimulation resulted in a clear progressive increase in C fibre response
292 latency (Fig. 1Biii), that was confirmed to be frequency-dependent, with 2Hz
293 stimulation resulting in greater ADS than 1Hz stimulation (P<0.0001, Fig. 1Ciii).
294 Notably, this C fibre ADS was significantly reduced by CFA inflammation,
295 independent of stimulation frequency (P<0.0001, Fig. 1Biii, Ciii).

296 To address the possibility that the observed CFA effect may reflect altered action
297 potential initiation time rather than altered conduction velocity slowing, compound
298 action potentials were recorded with the stimulating electrode placed at two different
299 positions on an individual dorsal root (Fig. 1Di). Subtraction of the C fibre latency
300 values recorded following stimulation at position 2 (short stimulation length) from
301 those stimulated at position 1 (long stimulation length) eliminates the contribution of
302 action potential initiation, leaving only conduction time (between the 2 stimulation
303 sites). This also revealed a progressive increase in C fibre response latency (Fig.
304 Dii) that was reduced by CFA inflammation independent of stimulus frequency
305 (P=0.009 Fig. 1Dii,iii). This suggests that altered action potential initiation time is not
306 therefore likely to account for the observed CFA inflammation dependent reduction in
307 C fibre ADS. Furthermore, the demonstration that subtracting the short from long
308 stimulation length results in a progressive increase in the C fibre response latency
309 confirms the length dependency of the phenomenon.

310 *CFA inflammation reduces ADS in monosynaptic C fibre input to lamina I NK1R+ neurons*

311 To explore the spinal impact of CFA reduced C fibre ADS, whole-cell patch-clamp
312 recordings were made from lamina I NK1R+ neurons, that are likely projection

313 neurons (Marshall et al., 1996; Todd et al., 2000; Spike et al., 2003; Al-Khater et al.,
314 2008) that receive monosynaptic input from both C and Aδ fibres (Torsney and
315 MacDermott, 2006; Torsney, 2011; Peirs et al., 2015). Comparison of the synaptic
316 transmission of these inputs, with their distinct afferent temporal relays (Fig. 1) will
317 provide insight into the central impact of ADS and its regulation by CFA. Lamina I
318 NK1R+ neurons were pre-identified using TMR-SP, which is not expected to alter
319 recorded synaptic activity (Tong and MacDermott, 2006), and has been employed
320 previously (Tong and MacDermott, 2006; Torsney and MacDermott, 2006; Torsney,
321 2011; Dickie and Torsney, 2014; Peirs et al., 2015).

322 Monosynaptic Aδ fibre eEPSCs were recorded, in spinal cord slices from control and
323 CFA treated rats (Fig. 2A), in response to dorsal root stimulation at frequencies of 1
324 and 2Hz. This resulted in a small, frequency dependent (P=0.016), progressive
325 increase in the eEPSC latency, that was unaffected by CFA inflammation (P=0.570,
326 Fig. 2B,C).

327 In contrast, repetitive stimulation of monosynaptic C fibre input to lamina I NK1R+
328 neurons resulted in a progressive increase in response latency, that was not altered
329 by stimulation frequency (P=0.521) but was markedly reduced by CFA inflammation
330 (P=0.013, Fig. 3) similar to the population C fibre CAP recordings. Notably, the initial
331 conduction velocity of monosynaptic C fibre input to lamina I NK1R+ neurons was
332 not altered by CFA inflammation (p=0.764, Mann-Whitney, data not shown), as
333 demonstrated previously (Torsney, 2011). CFA inflammation also did not alter the
334 initial peak amplitude of C fibre eEPSCs (p=0.568, Mann Whitney, data not shown),
335 as reported previously (Torsney, 2011; Dickie and Torsney, 2014) or the eEPSC
336 amplitude observed during repetitive stimulation (P=0.178, 2-way ANOVA, data not
337 shown). In summary, CFA inflammation does not alter the baseline CV of

monosynaptic C fibre inputs or their eEPSC peak amplitudes in lamina I NK1R+ neurons but it significantly reduces the progressive delay in synaptic transmission observed between C fibres and lamina I NK1R+ neurons.

CFA inflammation reduces both the average and range of ADS within the C fibre population

Longer stimulus trains and higher frequencies produce a more pronounced C fibre ADS that is associated with the occurrence of conduction failures (Thalhammer et al., 1994; Nakatsuka et al., 2000; Zhu et al., 2009) the prevalence of which can be altered in pain models (Sun et al., 2012; Wang et al., 2016a) which could additionally impact upon spinal pain processing. Therefore, to investigate the potential impact of conduction failures, longer stimulus trains and higher frequencies were employed.

In CAP recordings stimulating isolated dorsal roots with trains of 40 stimuli delivered at 1 and 2Hz resulted in a progressive increase in the C fibre response latency, which was frequency-dependent ($p<0.0001$) and was significantly reduced by CFA inflammation ($p=0.005$, Fig. 4A). It was not possible to employ higher frequency stimulation in the CAP recordings as the C fibre component of the compound action potential diminishes substantially during repetitive stimulation, presumably due to conduction failures, and as such could only be reliably quantified at 1 and 2 Hz. The change in C fibre response latency reflects the change in average conduction velocity of the population C fibre response. Given that different C fibres display different degrees of ADS (Thalhammer et al., 1994; Serra et al., 1999; Weidner et al., 1999) the change in width of the C fibre response was also measured (Fig. 4B), as this will reflect the change in range of conduction velocities present within the population and may therefore be a more informative measure of ADS across the entire C fibre population. 1 and 2Hz stimulation resulted in a progressive increase in the C fibre response width which was also regulated in a frequency and CFA

inflammation dependent manner (both p<0.0001, Fig. 4C). Notably, the initial width of the C fibre response was not significantly different between control and CFA tissue (p=0.27, unpaired t-test, data not shown).

CFA inflammation limits the progressive delay in synaptic transmission between C fibres and lamina I NK1R+ neurons without altering synaptic response failures.

In eEPSC recordings repetitive stimulation (x40) of the monosynaptic C fibre input to lamina I NK1R+ neurons at 2, 5 and 10Hz, resulted in a frequency-dependent ADS (P=0.039) that was reduced by CFA inflammation (p<0.0001, Fig. 5A). Plotting the percentage of lamina I NK1R+ neurons displaying synaptic response failures per stimulus number reveals a progressive increase in the number of failures (Fig. 5Bi-iii). This progressive increase in synaptic response failures is, as expected, frequency dependent, with the total no of failures per lamina I NK1R+ neuron increasing with stimulation frequency (p<0.0001). However, the total no of synaptic response failures was not affected by CFA inflammation (p=0.971, Fig. 5Biv).

C fibre ADS is regulated in a sex and inflammation dependent manner

To determine whether there is a sex difference in the ADS phenomenon CAP (Fig. 6A-C) and eEPSC (Fig. 6D-F) datasets were analysed for females and males separately. In CAP recordings from isolated dorsal roots a progressive increase in the latency and width of the population C fibre response was observed in both females (Fig. 6Ai,ii) and males (Fig. 6Bi,ii). AUC analysis reveals a significant interaction between sex and CFA inflammation for both latency change (Fig. 6Ci p=0.037) and width change (Fig. 6Cii p=0.003). ADS is more pronounced in control females and CFA inflammation reduces ADS, in females only, to a level that is now comparable with males for both latency change (CFA: female p=0.031; male

p=0.998) and width change measures (CFA: female p<0.0001; male p=0.525). Initial CV and CAP width were not altered in a sex or CFA inflammation dependent manner (CV: sex p=0.853, CFA p=0.120; width: sex p=0.482, CFA p=0.307, 2-way ANOVA data not shown).

Similarly, patch-clamp recordings from lamina I NK1R+ neurons show a progressive increase in the latency of monosynaptic C fibre evoked eEPSCs in both females (Fig. 6D) and males (Fig. 6E). Likewise AUC analysis reveals a significant interaction between sex and CFA inflammation (Fig.6F p=0.034). ADS is more pronounced in control females and CFA inflammation reduces ADS, in females only, to a level that is now comparable with males (CFA: female p=0.015; male p=0.996). However, baseline CV of monosynaptic C fibre inputs was not altered in a sex or CFA inflammation dependent manner (sex p=0.873; CFA p=0.923, data not shown).

Limiting ADS facilitates spinal summation

Our observations demonstrate that ADS results in a progressive delay in the synaptic transmission of C fibre input to individual spinal neurons. Moreover, given that repetitive C fibre stimulation results in a progressive increase in C fibre CAP width, that reflects the range of CVs within the population, ADS also likely reduces the temporal coincidence of population C fibre input at a spinal network level. Together, these findings suggest that ADS limits temporal summation of C fibre evoked synaptic activity at a spinal level. Thereby, reducing C fibre ADS should enhance spinal summation. To test this hypothesis we took advantage of the length dependency of ADS (Schmelz et al., 1995; Zhu et al., 2009) and assessed the impact of long (increased ADS) versus short (decreased ADS) root stimulation lengths upon spinal summation by recording cumulative dorsal root evoked ventral root potentials in a hemisected spinal cord preparation (Fig. 7A). Using this

412 preparation we are essentially able to assess the impact of ADS upon the circuitry
413 underlying the nociceptive flexion withdrawal reflex.

414 Repetitive stimulation of dorsal roots evoked a cumulative ventral root potential that
415 is blocked, as expected (Thompson et al., 1992) in the presence of the AMPA
416 receptor antagonist NBQX (10μM) and NMDA receptor antagonist APV (50μM, Fig.
417 7B). This spinal summation is frequency dependent (Fig. 7Ci), as previously reported
418 (Thompson et al., 1992). Interestingly, reducing the stimulation length strongly
419 facilitated the cumulative ventral root potential at 2Hz stimulation rates (Fig. 7Cii). To
420 quantify the degree of summation the amplitude of the cumulative ventral root
421 potential was measured 500ms following the last stimulus artefact and normalised to
422 the amplitude measured at 500ms following a single stimulus at long or short root
423 stimulation length to account for any potential difference in response amplitude
424 between stimulation lengths. Repeated measures ANOVA revealed a significant
425 interaction ($p=0.022$) between frequency ($p=0.039$) and length ($p=0.003$) dependent
426 effects and post tests revealed a significant difference between long and short
427 lengths at 2Hz stimulation rates only (Fig. 7Di, 2Hz $p<0.01$). Given the more
428 pronounced C-fibre ADS in females we predicted a greater change in facilitation of
429 spinal summation in females versus males. Indeed, there is a greater difference in
430 the % cumulative depolarisation between long and short stimulation length in
431 females at the 2Hz stimulation rate (Fig 7Dii, $p=0.028$).

432 To estimate the degree of change in ADS resulting from the average ~50% reduction
433 in root stimulation length in the DR-VRP recordings and thus the likely relevance of
434 the findings with respect to our sex/inflammation dependent changes in ADS we
435 revisited the x2 length stimulation CAP recordings displayed in Figure 1D. In the
436 CAP recordings a comparable reduction in stimulation length (~60%) reduced ADS

437 by ~20% as measured using AUC analysis of latency change (unpaired t-test
438 p=0.007, data not shown). Therefore in these DR-VRP recordings the short
439 stimulation length likely underestimates the average ~30% reduction in ADS
440 compared with the control female grouping (also assessed using AUC analysis of
441 latency change, Fig. 6Ci) underscoring the importance of these findings.

442 *Females display elevated noxious thermal thresholds and enhanced inflammatory thermal*
443 *hyperalgesia*

444 The demonstration that females display more pronounced ADS that is reduced by
445 CFA inflammation to a level that is comparable with males (control and CFA) along
446 with the observation that limiting ADS facilitates cumulative dorsal root evoked
447 ventral root potentials, the following predictions can be made regarding the
448 nociceptive flexion withdrawal reflex: 1) control females should have higher pain
449 thresholds than control males; 2) if other underlying inflammatory pain mechanisms
450 are not sex-dependent, then CFA inflammation reduced pain thresholds should be
451 similar between males and females and 3) because these predictions imply a greater
452 reduction in pain threshold in females vs males, females should display enhanced
453 inflammatory hypersensitivity.

454 Figure 8A-C displays behavioural data obtained in response to noxious radiant heat
455 stimuli in the CFA inflammation model that supports these predictions. Females
456 show increased inflammatory thermal hypersensitivity (Fig. 8B, p=0.032) that,
457 importantly, does not appear to be due to an increased inflammatory response
458 because CFA induced paw swelling is not significantly different between sexes (Fig.
459 8D, p=0.513). It instead reflects a larger reduction in thermal pain thresholds from an
460 elevated baseline level as can be observed by comparing contralateral male and
461 female withdrawal latencies (Fig. 8A). Notably, AUC analysis of thermal withdrawal

462 latency values (2-5d post CFA) reveals a significant interaction (p=0.044) between
463 sex and hindpaw (ipsilateral/contralateral) with post-tests revealing significantly
464 higher control/contralateral hindpaw values in females (p=0.022). The mechanical
465 threshold of the flexion withdrawal reflex was assessed using von Frey
466 monofilaments (Fig. 8E). AUC analysis reveals no significant interaction between sex
467 and hindpaw (repeated measures ANOVA p=0.180, data not shown). Furthermore,
468 inflammatory mechanical hypersensitivity was not altered in a sex-dependent
469 manner (Fig. 8F, p=0.548).

470 Discussion

471 We found that C fibre ADS is more pronounced in females than males and is
472 reduced by CFA inflammation in females only. This alters the timing of synaptic
473 transmission of monosynaptic C fibre input to lamina I NK1R+ neurons. Experimental
474 manipulation of ADS demonstrates it can influence spinal summation consistent with
475 observed sex differences in noxious thermal thresholds. We propose that ADS
476 regulates nociceptive drive to central pain circuits.

477 *Underlying mechanisms of ADS*

478 The underlying mechanism of ADS is not fully understood. It was initially proposed to
479 result from Na^+-K^+-ATPase dependent membrane hyperpolarisation (Rang and
480 Ritchie, 1968; Bostock and Grafe, 1985) but Na^+-K^+-ATPase blockade increased
481 rather than reduced ADS (De Col et al., 2008). Pharmacological studies then
482 suggested ADS reflects increased numbers of Nav channels entering a slow
483 inactivated state (De Col et al., 2008; Obreja et al., 2012). However modelling
484 suggested that while Nav channel slow inactivation contributes, ADS is most readily
485 associated with an increase in intra-axonal Na^+ concentration (Tigerholm et al.,

2014). Investigation of the differences between mechano-insensitive C fibres that display the greatest ADS versus mechano-sensitive C fibres that display minimal ADS (Weidner et al., 1999; Obreja et al., 2010) revealed that pronounced ADS was associated with more Nav1.8, less Nav1.7, more delayed rectifier potassium channel and less Na+/K+ ATPase, a profile consistent with enhanced accumulation of intracellular Na^+ (Petersson et al., 2014). Notably, given the observed sex/inflammation dependent ADS these molecules are regulated by peripheral inflammation (Coggeshall et al., 2004; Gould et al., 2004; Zhang and Nicol, 2004; Liang et al., 2013; Rahman and Dickenson, 2013; Waxman and Zamponi, 2014; Wang et al., 2015) but, despite being key pharmaceutical targets, sex differences in their regulation in C fibre nociceptors have not been explored.

In addition to the aforementioned molecules implicated in ADS there are many other ion channels/receptors whose expression, location and/or function can be altered by inflammation (Gold and Gebhart, 2010) and may contribute to altered ADS. Furthermore, previous studies have reported CFA inflammation induced changes in C-fibre nociceptor excitability including increased CV combined with reduced electrical thresholds in guinea-pig (Djouhri and Lawson, 2001) not replicated in this or other rat studies (Baba et al., 1999; Nakatsuka et al., 2000; Torsney, 2011), altered action potential shape (Djouhri and Lawson, 1999; Zhang et al., 2012) and spontaneous firing (Djouhri et al., 2006; Xiao and Bennett, 2007; Matson et al., 2015). Moreover, estrogen exacerbates inflammation increased excitability of temporomandibular joint afferents (Flake et al., 2005) and there are sex differences in inflammatory sensitisation of dural afferents (Scheff and Gold, 2011).

Evidence for altered ADS

Consistent with our observed inflammation altered ADS, the inflammatory mediator NGF reduces ADS in the mechano-insensitive C fibres that normally display pronounced ADS (Obreja et al., 2011) and is associated with less Nav1.8, more Nav1.7, less delayed rectifier potassium channel and more Na+/K+ ATPase (Petersson et al., 2014). Furthermore, there is reduced ADS in a diabetic neuropathic pain model (Wang et al., 2016b). In contrast, there is enhanced ADS in the spinal nerve ligation model of neuropathic pain (Shim et al., 2007) but these findings need to be considered within a partially denervated pain circuitry. Microneurography studies have also demonstrated altered ADS in chronic pain patients (Orstavik et al., 2003; Orstavik et al., 2006; Kleggetveit et al., 2012; Serra et al., 2014), interestingly, including patients with mutations in Nav1.7 (Namer et al., 2015) and Nav1.8 (Kist et al., 2016).

Physiological relevance of ADS

C fibre ADS is physiologically relevant because it occurs, not only following electrical stimulation, but also in response to natural stimulation of the skin (Thalhammer et al., 1994). Electrical stimulation induced ADS is useful as it can provide a readout of nociceptor excitability given the key roles of molecules implicated in ADS, such as Nav and HCN channels, in action potential generation and regulation of initial firing frequency in nociceptor terminals. It is not surprising therefore that altered ADS profiles are associated with changes in C fibre thresholds (Obreja et al., 2011), that ADS correlates with spontaneous firing (Kleggetveit et al., 2012) and is accompanied by a parallel increase in C fibre mechanical threshold (De Col et al., 2012).

Moreover, ADS is proposed to provide a memory trace of previous activity levels that can influence responses to subsequent inputs. Specifically, low level firing, comparable to spontaneous firing rates in inflammatory pain induces ADS that dynamically influences the response to higher frequency inputs such that they display reduced ADS or even speeding (Weidner et al., 2002). Importantly, in our data inflammation induced spontaneous C fibre firing will likely be absent, due to the lack of peripheral inflamed tissue, in our *ex vivo* preparations. Therefore our observations may well underestimate the degree of inflammation reduced ADS as our recorded data likely only reflects the inflammation altered expression levels of ion channels involved in ADS and not the dynamic regulation of ADS by ongoing C fibre activity.

Here we demonstrate, that in addition to ADS providing a nociceptor excitability readout and a dynamic memory, that the progressive slowing per se significantly influences the relay of nociceptive signals such that the firing frequency initiated in the periphery is not faithfully transmitted to the spinal cord. In individual spinal neurons there is a progressive delay in synaptic transmission of C fibre inputs and we also observe a progressive reduction in temporal coincidence of population C fibre input that we predicted should alter summation at a spinal network level. Decreasing stimulation length in DR-VRP recordings demonstrated that reducing ADS did indeed facilitate spinal summation. Interestingly, facilitation was only significant at the 2Hz stimulation rate and strikingly, increased summation to a level comparable with that observed at 10Hz. The lack of effect at higher frequencies probably reflects the increased incidence of synaptic failures with increasing stimulation frequency (see Figure 5B) likely resulting from frequency dependent C

560 fibre conduction failure (Nakatsuka et al., 2000; Zhu et al., 2009; Sun et al., 2012;
561 Wang et al., 2016a) that will limit the maximal extent of spinal summation. Notably,
562 spontaneous firing rates resulting from tissue inflammation are typically <1Hz
563 (Djouhri et al., 2006; Xiao and Bennett, 2007; Matson et al., 2015) whereas noxious
564 stimuli evoke firing in the 1-10Hz range (Lynn and Carpenter, 1982; Leem et al.,
565 1993). We therefore propose, that reducing ADS thereby promoting temporal
566 coincidence of nociceptive input, as a novel mechanism of hyperalgesia whereby
567 lower intensity noxious inputs can be amplified but high intensity inputs are not
568 further amplified due to transmission failures which may reflect an intrinsic self-
569 inhibition mechanism to limit overdrive of the nociceptive pathways. Notably ADS
570 was altered in the monosynaptic C fibre input to lamina I NK1R+ likely projection
571 neurons that display tightly controlled spike timing-dependent plasticity (Li and
572 Baccei, 2016). Therefore these changes in ADS may influence the involvement of
573 these output neurons in synaptic plasticity (Ikeda et al., 2003; Ikeda et al., 2006)
574 spinal supra-spinal loop activity (Suzuki et al., 2002) and chronic pain (Mantyh et al.,
575 1997; Nichols et al., 1999). Finally promotion of closely timed nociceptive inputs
576 appears to be a feature following injury with the recent demonstration of novel DRG
577 neuronal co-activation following CFA inflammation that contributes to mechanical
578 hyperalgesia (Kim et al., 2016).
579

580 The pronounced ADS in control females that was reduced by CFA to male levels,
581 along with the observation that limiting ADS facilitates spinal summation, was
582 consistent with behavioural observations of elevated noxious thermal latencies and
583 enhanced inflammatory thermal hyperalgesia in females. While the enhanced
584 hypersensitivity is consistent with sex differences in human studies the elevated

noxious thermal latencies are not (Mogil, 2012; Bartley and Fillingim, 2013). However, we studied spinal reflex behaviours in which the afferent input is a major component of the underlying neural circuitry whereas human subjective pain scoring additionally involves higher level cognitive processing. If differing degrees of ADS contributes to sex differences in pain thresholds, it is not surprising that this is specific for thermal vs mechanical sensitivity because the inflammatory mediator NGF can reduce ADS in mechano-insensitive C fibres that can respond to heat (Schmidt et al., 1995; Schmelz et al., 1997) without unmasking of mechanical sensitivity (Obreja et al., 2011). However, the lack of sex differences in mechanical sensitivity may reflect the use of von Frey hairs to identify mechanical thresholds rather than a noxious mechanical stimulus. Alternatively it may be that different peripheral mechanisms, DRG neuronal coupling and reduced ADS are employed to augment noxious mechanical and thermal inputs, respectively. It will be important to determine the extent to which ADS regulates the processing of noxious stimuli *in vivo* and also identify the mechanisms underlying sex dependent ADS and whether these are controlled by sex hormones or genetic factors.

It has been long established that the intensity of a sensation is encoded by afferent firing frequency. Here we propose that the firing frequency initiated in the periphery, is diminished en route to the CNS, by ADS in nociceptive C fibres and that inflammation/sex dependent regulation of ADS can thereby peripherally modulate spinal processing and pain sensation.

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Figure Legends

Figure 1

CFA inflammation reduces C fibre activity-dependent slowing (ADS) in isolated dorsal roots. **Ai** Two suction electrodes were used to stimulate and record compound action potentials from L4/L5 dorsal roots. Representative compound action potentials recorded from dorsal roots isolated from control (**Aii**) and CFA (**Aiii**) treated rats, illustrating the fast (Aβ), medium (Aδ) and slow (C) conducting components. The x16 traces recorded in response to 2Hz dorsal root stimulation are shown. Arrowheads indicate the stimulus artefact. Inserts magnify the C fibre component with broken lines indicating the negative peaks of the first and last responses. Repetitive stimulation of dorsal roots at 2Hz (**B**) results in a negligible change in the latency of Aβ (**i**) or Aδ fibre (**ii**) responses whereas C fibres display a progressive increase in response latency (**iii**). **C** AUC analysis of latency change reveals that CFA inflammation reduces the frequency dependent progressive latency change observed in C fibres (2-way ANOVA, CFA $p<0.0001$; frequency $p<0001$). **D** Eliminating the contribution of action potential initiation time to latency change, by subtracting the latency values obtained at the short (position 2) from the long (position 1) root stimulation length **(i)** confirms that CFA attenuates C fibre ADS **(ii, iii)** (2-way ANOVA, CFA $p<0.0001$). Data in **B**, **C**: Aβ control n=10, CFA n=12; Aδ/C control n=11, CFA n=13. Data in **D**; control n=10, CFA n=12.

Figure 2

CFA inflammation does not alter limited ADS in monosynaptic Aδ fibre input to lamina I NK1R+ neurons. **Ai** eEPSCs were recorded from pre-labelled lamina I NK1R+ neurons, in spinal cord slices in response to stimulation of attached dorsal roots. Representative monosynaptic Aδ fibre eEPSCs recorded in tissue isolated from control (**Aii**) and CFA (**Aiii**) treated rats. Each trace comprises 16 traces recorded in response to dorsal root stimulation at 2Hz. Broken lines and arrows denote the latency of the first and last trace, measured as the time between the stimulus artefact (denoted by the arrowhead) and the onset of the monosynaptic response. **B** Stimulation of monosynaptic Aδ fibre input to lamina I NK1R+ neurons at 1 (**i**) or 2Hz (**ii**) results in a small degree of ADS. **C** AUC analysis of latency change reveals that CFA inflammation does not affect the small frequency dependent progressive latency change (2-way ANOVA, CFA p=0.570; frequency p=0.016). 1Hz: control n=7, CFA n=12; 2Hz: control n=13, CFA n=22. Note that scaling in B and C is identical to that in Figure 3.

Figure 3

CFA inflammation reduces ADS in monosynaptic C fibre input to lamina I NK1R+ neurons. **A** Representative monosynaptic C fibre eEPSCs recorded in tissue isolated from control (**i**) and CFA (**ii**) treated rats. Each trace comprises 16 traces recorded in response to repetitive dorsal root stimulation at 1Hz. Broken lines and arrows denote the latency of the first and last trace, measured as the time between the stimulus artefact (denoted by the arrowhead) and the onset of the monosynaptic response. **B** Stimulation of monosynaptic C fibre input to lamina I NK1R+ neurons at 1 (**i**) or 2Hz

(**ii**) results in a progressive increase in response latency. AUC analysis of latency change **(C)** reveals that CFA attenuates ADS but it is not affected by stimulation frequency (2-way ANOVA, CFA p=0.013; frequency p=0.521). 1Hz: control n=42, CFA n=64; 2Hz: control n=16, CFA n=36.

Figure 4

CFA inflammation reduces the average and range of ADS within the C fibre population. **A** C fibre compound action potentials recorded in response to trains of 40 stimuli delivered at 1 (**i**) or 2Hz (**ii**) results in a progressive slowing of the response latency. AUC analysis of latency change **(iii)** reveals that CFA inflammation reduces the frequency dependent progressive latency change (2-way ANOVA, CFA p=0.005; frequency p<0001). **B** Representative C fibre compound action potentials recorded, in response to 2Hz stimulation, from dorsal roots isolated from control (**i**) and CFA (**ii**) treated rats, with broken lines and arrows indicating the width (positive-positive peak) of the first and last response. **C** Trains of 40 stimuli delivered at 1 (**i**) or 2Hz (**ii**) result in a progressive increase in C fibre compound action potential width. AUC analysis of width change **(iii)** reveals that CFA inflammation reduces the frequency dependent progressive width increase (2-way ANOVA, CFA p<0.0001; frequency p<0001). All: control n=18, CFA n=16.

Figure 5

CFA inflammation reduces ADS in monosynaptic C fibre input to lamina I NK1R+ neurons during high frequency stimulus trains but does not alter synaptic response failures. **A.** Repetitive stimulation of monosynaptic C fibre input to lamina I NK1R+ neurons, using trains of 40 stimuli, delivered at 2 (**i**), 5 (**ii**) or 10Hz (**iii**) results **(iv)** in

a frequency dependent progressive increase in the eEPSC latency, that is significantly reduced by CFA inflammation (2-way ANOVA, CFA p<0001; frequency p=0.039). **B.** There is a progressive increase in the number of synaptic response failures per stimulus number during stimulation of monosynaptic C fibre input to lamina I NK1R+ neurons at 2 (**i**), 5 (**ii**) or 10Hz (**iii**). The total number of synaptic response failures **(iv)** is frequency dependent but not altered by CFA inflammation (2-way RM ANOVA, CFA p=0.971; frequency p<0.0001). All groups n=9.

Figure 6

CFA inflammation reduces ADS, within the C fibre population and in the monosynaptic C fibre input to lamina I NK1R+ neurons, in females only. C fibre compound action potentials recorded in response to 2Hz trains (x40 stimuli) in females **(A)** and males **(B)** results in a progressive slowing of the response latency **(i)** and width **(ii)**. **C** AUC analysis of latency change **(i)** and width change **(ii)** reveals a significant interaction between sex and inflammation (2-way ANOVA latency change p=0.037, width change p= p=0.003) with CFA inflammation reducing ADS in females only (latency change females p=0.031, males p=0.998; width change females p<0.0001, males p=0.525, Tukey multiple comparisons test). Monosynaptic eEPSCs in lamina I NK1R+ neurons recorded in response to 2Hz trains (x40 stimuli) results in a progressive slowing of the response latency in females **(D)** and males **(E)**. **F** AUC analysis of latency change reveals a significant interaction between sex and inflammation (2-way ANOVA p=0.034) with CFA inflammation reducing ADS in females only (female p=0.015; male p=0.996, Tukey multiple comparisons test). CAP: female control n=9, female CFA n=7, male control n=9, male CFA n=9. eEPSC: female control n=10, female CFA n=17, male control n=6, male CFA n=16.

Figure 7

Cumulative dorsal root evoked ventral root potentials are regulated in a frequency and length dependent manner. **A** Ventral root potentials were recorded from a hemisected spinal cord preparation in response to long length and short length dorsal root stimulation (x20) to increase and decrease the spinal impact of ADS, respectively, using suction electrodes. **B** Representative cumulative dorsal root evoked (5Hz) ventral root potential **(i)** that is abolished in the presence of NBQX (10μM) and APV (50μM) **(ii)**. Stimulus artefact visible as vertical lines in all traces. **C** Representative cumulative dorsal root evoked ventral root potentials induced by 2, 5 and 10Hz stimulation over long **(i)** and short **(ii)** dorsal root lengths. Arrows denote the 500ms timepoint, following the last stimulus artefact, when amplitude is measured. **D(i)** Repeated measures ANOVA of normalised cumulative ventral root potentials reveals both frequency ($p=0.039$) and length ($p=0.003$) dependent effects that significantly interact ($p=0.022$) with Sidak's multiple comparisons test revealing a significant impact of length at 2Hz only ($p<0.01$), n=10. **(ii)** Difference in % cumulative depolarisation between long and short stimulation lengths is significantly greater in females (One-tailed Mann-Whitney test, $p=0.028$), n=5 both groups.

Figure 8

Behavioural analysis of the nociceptive flexion withdrawal reflex demonstrates elevated thermal pain thresholds and enhanced inflammatory thermal hyperalgesia in females. **A** Ipsilateral and contralateral hindpaw withdrawal latencies to noxious radiant heat stimuli (Hargreaves) were measured in female and male rats prior to and 2-5d following CFA intraplantar injection. **B** Ipsilateral withdrawal latency values expressed as a percentage of contralateral values, reveal a sex difference in thermal

hyperalgesia (repeated measures-ANOVA, p=0.032). **C** AUC analysis (2-5d post CFA) shows a significant sex difference in contralateral hindpaw withdrawal latency with higher values in females (2-way ANOVA followed by Sidak's multiple comparisons test, p=0.022). **D** Ipsilateral hindpaw thickness is expressed as a percentage of contralateral values and demonstrates that CFA-induced paw swelling is not sex dependent (repeated measures ANOVA, p=0.513). **E** Ipsilateral and contralateral hindpaw mechanical thresholds (von Frey) were also measured in the same female and male rats prior to and 2-5d following CFA intraplantar injection. **F** Ipsilateral mechanical thresholds are expressed as a percentage of contralateral values and demonstrates that mechanical inflammatory hypersensitivity is not sex dependent (repeated measures ANOVA, p=0.548). All graphs female n=9, male n=7.